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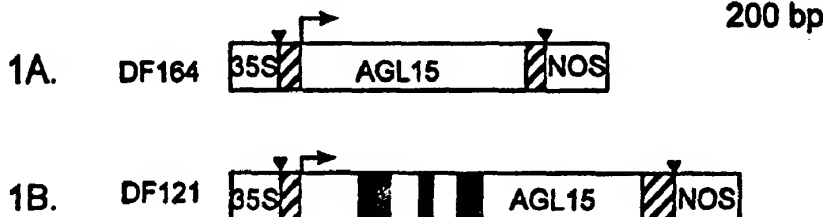
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(54) Title: AGL15 SEQUENCES IN TRANSGENIC PLANTS



(57) Abstract

A transgenic flowering plant exhibiting a novel phenotype contains in its genome a genetic construct in which an AGL15 sequence is placed under the control of a promoter that is expressed in the plant, the promoter not being natively associated with the AGL15 sequence. A genetic construct that is useful for obtaining transgenic plants includes an AGL15 sequence under the control of a promoter, not natively associated with the AGL15 sequence, which is functional in plants.

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## 5 AGL15 SEQUENCES IN TRANSGENIC PLANTS

## CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority from provisional application number 60/031,205 filed November 21, 1996.

## STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

10 This invention was made with United States Government support through NSF grant # DCB-9105527, NSF Postdoctoral Research Fellowship grant # BIR-9403929 awarded to Sharyn E. Perry, and grant # BIR-92020331 from the DOE/NSF/USDA Collaborative Program on Research in Plant Biology Training  
15 Program. The United States Government has certain rights in this invention.

## BACKGROUND OF THE INVENTION

Modern biotechnology has devoted considerable effort to the development of phenotypically distinct plants with  
20 economically advantageous qualities. Valuable features in food crops include increased yields, extended shelf-life, and delayed fruit ripening that is susceptible to external control. In the floral industry, there is interest in delaying senescence of both cut and uncut flowers.

25 Efforts to develop crop plants that produce higher yields have been directed toward pest control or toward the selection and breeding of varieties that bear greater numbers of fruits, or that produce larger fruits. These crop breeding endeavors are very time-consuming and labor-intensive, and have not  
30 resulted in dramatically increased crop yields.

Much of the research on senescence in plants has focused on the manipulation of the plant hormone cytokinin, because there is evidence that suggests an inverse correlation between

levels of the plant hormone cytokinin and the onset of senescence. Plant varieties with high levels of endogenous cytokinin tend to have blooms that are longer lived. The application of cytokinin to blooms or to the holding solution of cut flowers has been tested as a means for extending flower longevity. The success of this method is equivocal, and plant response to cytokinins is affected by numerous parameters, some of which are immutable.

One of the means by which cytokinin is thought to delay floral senescence is by decreasing the sensitivity of floral tissues to ethylene and/or interfering with the production of ethylene. Increased levels of ethylene are correlated with accelerated senescence in petals. Experiments designed to manipulate ethylene levels were conducted using transgenic carnations that contained a construct directing expression of an antisense RNA complementary to the mRNA of ACC synthase, an enzyme involved in the biosynthesis of ethylene. The results of that research did not conclusively demonstrate delayed senescence in flowers of transgenic carnations in which the antisense RNA was expressed.

In fruits, high levels of cytokinins are associated with delayed ripening, but not delayed senescence. The exogenous application of cytokinins to ripening fruit has been employed to delay ripening. US Patent No. 5,177,307 describes the manipulation of cytokinins in transgenic tomato plants containing a construct that directs the tissue-specific expression of an enzyme involved in the biosynthesis of cytokinin. These transgenic tomato plants exhibit increased expression of cytokinins, and produce fruit with a blotchy appearance.

Tillable land available for production of food crops continues to diminish because each year, more acreage is devoted to alternative uses. At the same time, the human population is rapidly increasing. Therefore, it is essential to increase agricultural productivity to meet the nutritional needs of the world's burgeoning population.

- Within the floral and landscaping industries, producers, florists, and professional gardeners and landscapers are desirous of methods for increasing the number and persistence of blooms on ornamental flowering plants and cut flowers.
- 5 Human enjoyment of ornamental flowering plants and cut flowers can be enhanced by extending the longevity of the flowers.

#### BRIEF SUMMARY OF THE INVENTION

- 10 The present invention is a transgenic flowering plant comprising in its genome a genetic construct comprising an AGL15 (AGL for AGAMOUS-like) DNA sequence and a promoter, not natively associated with the AGL15 sequence, that promotes expression of the AGL15 sequence in the plant.

- 15 The present invention is also a plant cell, derived from a flowering plant, comprising in its genome a genetic construct comprising an AGL15 DNA sequence and a promoter, not natively associated with the AGL15 sequence, that promotes gene expression in plants.

- 20 The present invention is also a seed, derived from a flowering plant, comprising in its genome a genetic construct comprising an AGL15 DNA sequence and a promoter, not natively associated with the AGL15 sequence, that promotes gene expression in plants.

- 25 The present invention is also a genetic construct comprising an AGL15 DNA sequence and a promoter, not natively associated with the AGL15 sequence, that promotes expression of the AGL15 sequence in plants.

It is an object of the present invention to provide a transgenic flowering plant that has a novel phenotype with advantageous properties.

- 30 It is another object of the present invention to provide a transgenic seed from flowering plants.

- 35 It is an object of the present invention to provide a genetic construct comprising an AGL15 sequence and a promoter, not natively associated with the AGL15 sequence and which promotes expression of AGL15 in plants at levels that result in novel phenotypes.

Other objects, advantages, and features of the present invention will become apparent after review of the specification, drawings, and claims.

#### BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

5 Fig. 1A is a schematic map of a genetic construct, designated DF164, which contains the cauliflower mosaic virus 35S promoter (35S), an Arabidopsis AGL15 cDNA fragment (SEQ ID NO:1) comprising an 18-bp 5' untranslated region (UTR), an 807-bp open reading frame (ORF), a 245-bp 3' UTR, and a nopaline  
10 synthetase terminator (NOS). The inverted triangles demark the AGL15 cDNA fragment; the crosshatched regions indicate the 5' and 3' UTRs; the white region denotes the AGL15 ORF; the arrow indicates the translational start site and the direction in which the sequence is read.

15 Fig. 1B is a schematic map of a genetic construct, designated DF121, which contains the sequence of DF164 and three introns from a genomic Arabidopsis AGL15 gene that were introduced into DF164 by genetic engineering methods known in the art. The symbols and shadings are employed in Fig. 1A have  
20 the same meanings in Fig. 1B. Additionally, the solid regions within the ORF denote introns derived from the Arabidopsis genomic AGL15 sequence.

#### DETAILED DESCRIPTION OF THE INVENTION

One aspect of the present invention is a transgenic  
25 flowering plant that contains in its genome a genetic construct comprising an AGL15 DNA sequence and a promoter, not natively associated with the AGL15 sequence, which promotes expression of the AGL15 in the transgenic flowering plant.

As an example of the efficacy of this invention,  
30 transgenic Arabidopsis plants that contain a genetic construct comprising an AGL15 sequence under the control of the cauliflower mosaic virus 35S promoter (CaMV 35S) have been developed as detailed in the examples below. Arabidopsis plants in which the recombinant AGL15 sequence is expressed  
35 exhibit unique phenotypes, characterized by a number of advantageous qualities, including increased numbers of flowers

and fruits, delayed maturation of fruit, delayed floral organ senescence and abscission, and delayed senescence of cut flowers and inflorescences.

5 As the examples below demonstrate, AGL15 sequences are ubiquitous and highly conserved among angiosperm plant species. It is therefore expected that any flowering plant can be used in the practice of the present invention. For example, a flowering plant that produces edible fruit may be used. The flowering plant could also be a plant whose flowers are valued  
10 for their ornamental properties. The present invention could be practiced using a flowering plant that is raised for its production of seed, flowers, or fruit.

Transgenic Arabidopsis plants were obtained using the Agrobacterium transformation system, as described in the  
15 examples. Agrobacterium-mediated transformation is known to work well with all dicot plants and some monocots. Other methods of transformation equally useful in dicots and monocots may also be used in the practice of the present invention. Transgenic plants may be obtained by particle bombardment,  
20 electroporation, or by any other method of transforming plants known to one skilled in the art of plant molecular biology. The experience to date in the technology of plant genetic engineering is that the method of gene introduction is not of particular importance in the phenotype achieved in the  
25 transgenic plants.

A transgenic plant may be obtained directly by transformation of a plant cell in culture and regeneration of a plant. More practically, transgenic plants may be obtained from transgenic seeds set by parental transgenic plants.  
30 Transgenic plants pass on inserted genes, sometimes referred to as transgenes, to their progeny by normal Mendellian inheritance just as they do their native genes. Methods for breeding and regenerating plants of agronomic interest are known in the art.

35 Two AGL15 sequences derived from Arabidopsis have been found to be useful in the practice of the present invention. One useful sequence is an Arabidopsis AGL15 cDNA sequence (SEQ

ID NO:1) that has been isolated and characterized as described in detail in the examples. Briefly, the Arabidopsis AGL15 cDNA was derived from mRNA that is preferentially expressed during embryogenesis. A second useful Arabidopsis AGL15 sequence was made by genetically engineering the cDNA sequence of SEQ ID NO:1 to include three introns from the sole Arabidopsis genomic AGL15 gene sequence, which was isolated as described below.

The examples below demonstrate that other plants contain sequences that are homologous to the AGL15 sequence of Arabidopsis. Two Brassica napus AGL15 cDNA sequences and one genomic sequence have been identified and characterized as described in the examples below. DNA sequence analysis revealed that these sequences are highly homologous to the Arabidopsis AGL15 gene.

Numerous genera of flowering plants were examined and found to produce a protein product that binds antibodies raised against an AGL15-specific polypeptide. By "AGL15 sequence" it is meant a DNA sequence sufficiently homologous to SEQ ID NO:1 to exhibit AGL15 activity when expressed in a transgenic plant under the control of a promoter functional in that plant. An AGL15 sequence may be an unmodified sequence isolated from any flowering plant, a cDNA sequence derived from mRNA preferentially expressed during embryogenesis, a cDNA sequence engineered to include introns, a sequence that is modified in vitro to contain a sequence distinct from that of a naturally occurring sequence, a heterologous sequence that is synthesized in vitro.

By "AGL15 activity" it is meant the occurrence of a novel phenotype, characterized by increased numbers of flowers and fruits, delayed maturation of fruit, delayed floral organ senescence and abscission, or delayed senescence of cut flowers and inflorescences, which correlates with the expression of an AGL15 sequence in a transgenic plant comprising in its genome the AGL15 sequence under the control of a functional promoter that is not natively associated with the AGL15 sequence.



B cause AGL15 sequences are highly conserved among flowering plants, it is reasonably anticipated that an AGL15 sequence from any flowering plant may be used in the practice of the present invention. To identify potential AGL15 sequences, which are preferentially expressed during embryogenesis, an AGL15-specific region of an AGL15 sequence may be used to probe a cDNA library made from plant embryos. Another approach to identifying AGL15 sequences employs PCR amplification using AGL15-specific degenerate primers. In addition, AGL15 sequences may be identified in a plant genomic library using an AGL15-specific probe.

Sequences homologous to AGL15-specific sequences from Arabidopsis have been found in numerous species of flowering plants. It is anticipated that these sequences have AGL15 activity, even if they do not exhibit complete sequence identity with SEQ ID NO:1. It is expected that polyploid plants having more than one copy of the AGL15 gene may have allelic variations among AGL15 gene sequences. It is anticipated that putative AGL15 sequences having less than 100% sequence homology to the sequence shown in SEQ ID NO:1 will exhibit AGL15 activity.

It is envisioned that minor sequence variations from SEQ ID NO:1 associated with nucleotide additions, deletions, and mutations, whether naturally occurring or introduced in vitro, will not affect AGL15 activity. The scope of the present invention is intended to encompass minor variations in AGL15 sequences.

It is anticipated that a region of an AGL15 cDNA sequence may be used to construct a heterologous sequence having AGL15 activity using methods known in the art of molecular biology. This may be accomplished by ligating an AGL15-specific region of an AGL15 sequence to a DNA sequence that encodes a protein that lacks AGL15 activity, but which has domains that are functionally analogous to domains encoded by nonAGL15-specific regions of an AGL15 sequence.

By an "AGL15-specific sequence", it is meant a DNA sequence that is common to all putative AGL15 sequences and

which is distinct from sequences common to both AGL15 and related protein-coding sequences that lack AGL15 activity. Characterization of protein domains encoded by AGL15 sequences is discussed in detail in the examples. Briefly, an AGL15 protein contains a domain that is unique to AGL15, as well as domains that are common to many related proteins not known to possess AGL15 activity. The sequence comprising bases 190-1060 of SEQ ID NO:1 is an example of an AGL15-specific sequence.

The present invention is also directed toward a genetic construct comprising the DNA sequence and a promoter, not naturally associated with the AGL15 sequence, which promotes expression of the AGL15 sequence in plants at levels sufficient to cause novel phenotypes. The creation of two constructs that contain one of the constructs in Arabidopsis plants that are shown in Fig. 1A and Fig. 1B. Briefly, relevant features of these constructs include, in 5' to 3' order, the CaMV 35S promoter operably connected to the AGL15 sequence of SEQ ID NO:1, or SEQ ID NO:1 modified to include three genomic introns, a protein that confers kanamycin (NOS), and a gene that encodes the nopaline synthase terminator (NOS).

The CaMV 35S promoter is a constitutive promoter known to function in a wide variety of plants. Other promoters that are functional in the plant into which the construct will be introduced may be used to create genetic constructs to be used in the practice of the present invention. These may include other constitutive promoters, tissue-specific promoters, developmental stage-specific promoters, and inducible promoters. Promoters may also contain certain enhancer sequence elements that improve the efficiency of transcription.

The AGL15 sequence used to construct DF164 is an Arabidopsis cDNA sequence that contains a complete ORF, as well as 5' and 3' UTRs. A suitable genetic construct may contain AGL15 cDNA or genomic sequences from other genera of plants. A suitable construct may include a complete AGL15 ORF, with or

without a 5' UTR, and with or without a 3' UTR. The length of any UTR that is included in a construct may vary. A suitable construct may include an AGL15-specific subregion of an AGL15 ORF. It is anticipated that a construct that includes an AGL15-specific subregion ligated in-frame to a heterologous sequence that encodes the nonAGL15-specific domains of the AGL15 protein may be used in the practice of the present invention.

The examples below demonstrate that the construct DF121, which contains the Arabidopsis cDNA sequence of SEQ ID NO:1, into which three genomic introns have been engineered, is useful in the practice of the present invention. In general, genomic introns enhance expression of gene sequences. It has also been demonstrated that DF164, a construct containing an AGL15 sequence with no introns, works in the practice of the present invention. It is therefore reasonable to expect that a construct containing an AGL15 sequence with one or two introns may also be used to generate transgenic plants with advantageous features. It is anticipated that a construct containing an AGL15 sequence with more than three introns may be used in the present invention.

The examples below describe the use of an expression vector that contains a kanamycin resistance gene as a selectable marker for selection of plants that have been transformed with the genetic construct. Numerous selectable markers, including antibiotic and herbicide resistance genes, are known in the art of plant molecular biology and may be used to construct expression vectors suitable for the practice of the present invention. Expression vectors may be engineered to include screenable markers, such as beta-glucuronidase (GUS).

The genetic constructs employed in the examples below were engineered using the plasmid vector pBI121 (Clontech). It is anticipated that other plasmid vectors or viral vectors, or other vectors that are known in the art of molecular biology, will be useful in the development of a construct that may be used to transform a plant and allow expression of an AGL15 sequence. We describe the creation of a genetic construct

suitable for transformation using the Agrobacterium system. However, any transformation system for obtaining transgenic plants, including particle bombardment, electroporation, or any other method known in the art, may be employed in the practice of the present invention. The construction of vectors and the adaptation of a vector to a particular transformation system are within the ability of one skilled in the art.

The nonlimiting examples that follow are intended to be purely illustrative. Publications cited below are incorporated by reference herein.

#### EXAMPLES

##### Isolation and Characterization of AGL15 Sequences

Genes that are preferentially expressed during embryogenesis in *Brassica napus* were identified using the differential display method of Liang and Pardee (Science 257:967-971, 1992). *Brassica* was chosen for initial isolation of sequences preferentially expressed during embryogenesis because of the relatively large size of *Brassica* embryos. Using the differential display method, mRNA sequences present in developing embryos of *Brassica napus* at the transition and heart stages were compared with mRNA sequences present in older embryos, the post-germination shoot apex, and mature leaves.

One microgram of total RNA from each sample was used in the first strand synthesis reaction. Polymerase chain reaction (PCR) was performed using one-tenth of the first strand cDNA reaction mixture, various primer sets, and 35 S-dATP in 20- $\mu$ l reactions. After 40 amplification cycles (94 °C for 30 sec, 42 °C for 1 min, and 72 °C for 30 sec), a 4  $\mu$ l aliquot of the reaction mixture was loaded onto a 6% polyacrylamide sequencing gel. Following electrophoresis, the gel was dried and the differential bands were visualized using autoradiography.

One amplification product, derived from the priming oligonucleotides 5'-T<sub>12</sub>CG-3' and 5'-GAGCTGAAC-3', was present only in samples from developing embryos. This amplification product of approximately 500 bp was recovered by excision of the corresponding band from the dried gel, rehydration of the

excised gel band, and electroelution of the cDNA product from the gel. The cDNA was ligated to pBluescript SK- (Stratagene) vector DNA that had been digested with EcoRV and tailed with a single thymidine residue using Taq polymerase. The 500 bp  
5 insert was used to screen a cDNA library prepared from transition stage (16-19 days after pollination) *B. napus* embryos. Ten positive clones were identified.

Sequences from several of the ten isolated cDNA clones were analyzed. The full-length Brassica cDNA sequence (SEQ ID  
10 NO:2) has an open reading frame of 795 bp and encodes a predicted 30-kD protein of 264 amino acid residues (SEQ ID NO:3). Protein data base comparisons indicate strong homologies to a family of both known and putative transcriptional regulators, known as MADS domain proteins  
15 (Schwarz-Sommer et al., Science 250:931-936, 1990). Members of the MADS domain family have been demonstrated to play key roles in critical developmental events in diverse eukaryotic organisms, including yeast, arthropods, vertebrates, and plants.

20 In general, the MADS domain regulatory proteins possess a MADS domain, which is a highly conserved region of 55-60 amino acid residues that includes a DNA binding domain, a dimerization domain, and a putative phosphorylation site for calmodulin-dependent protein kinases (Sommer et al. EMBO J.  
25 9:605-613, 1990). The MADS domain occurs on the N-terminal region of regulatory protein sequences. Members of the MADS domain family of transcriptional regulators have a second region in common, designated the K domain. The K domains exhibit less conservation of primary sequence but share a  
30 putative amphipathic  $\alpha$ -helical structure that may be involved in facilitating protein-protein interactions. The C-terminal regions of MADS domain regulatory proteins are divergent.

The *B. napus* MADS domain gene was subsequently designated AGL15 in accordance with the numbering scheme of Rounsley et al. (Plant Cell 7:1259-1269, 1995). Because this species of  
35 Brassica is tetraploid, it is expected that there is more than one AGL15 locus in the *B. napus* genome. The first cDNA

species that was characterized was designated *B. napus* AGL15-1. A genomic AGL15-1 sequence from Brassica was isolated from a genomic library using a probe downstream of the highly conserved MADS domain of the Brassica AGL15-1 cDNA. The sequence of the genomic AGL15-1 sequence from Brassica is shown in SEQ ID NO:4. A second Brassica AGL15 cDNA species, designated AGL15-2, was identified. Its sequence is shown in SEQ ID NO:5.

A homolog of the *B. napus* AGL15-1 in *Arabidopsis thaliana* was identified by probing an *Arabidopsis thaliana* cDNA library from developing siliques with a sequence from *B. napus* AGL15-1 downstream of the MADS domain. Several full-length cDNA clones were identified. The *Arabidopsis* homolog of AGL15-1 is shown in SEQ ID NO:1. A region downstream of the MADS domain of the *Arabidopsis* AGL15 cDNA sequence was used to probe an *Arabidopsis* genomic library to identify a genomic clone. The DNA sequence of the *Arabidopsis* genomic AGL15 sequence was determined and is shown in SEQ ID NO:6.

A comparison of the predicted amino acid sequences encoded by the AGL15 cDNA sequences of Brassica (SEQ ID NO:3) and *Arabidopsis* (SEQ ID NO:7) revealed that the putative transcription factors share 95% amino acid identity in the MADS domain, 71% in the K domain, and 75% in the C-terminal region.

A comparison of protein-coding regions of the AGL15 cDNA sequences from *Arabidopsis* and Brassica revealed that the *Arabidopsis* AGL15 cDNA sequence contains an insertion of 4 bases in the C-terminal region. The insertion causes in a frameshift mutation relative to AGL15-1 and the addition of 16 amino acid residues not present in the Brassica protein. Alignment and comparison of the DNA sequences in the C-terminal coding regions of the genes was performed after introducing a four-base gap in the region of AGL15-1 corresponding to the 4-base insertion in the *Arabidopsis* sequence. This comparison revealed 100% homology between the AGL15 protein-coding sequences of Brassica and *Arabidopsis*, exclusive of the four-base insert. (Heck et al. Plant Cell 7:1271-1282, 1995).

Genomic DNA blot analysis and low-stringency hybridizations suggest that AGL15 represents a single locus in *Arabidopsis*. Evidence that transcripts of the AGL15 gene are present in developing embryos is provided by reverse transcription-PCR using isolated *Arabidopsis* embryos (Heck and Fernandez, unpublished results) and by *in situ* hybridization (Rounsley et al., Plant Cell 7:1259-1269, 1995).

The AGL15 gene is one of 24 members of the MADS domain genes that have been isolated from *Arabidopsis*. The AGL15 gene is the only *Arabidopsis* MADS domain regulatory factor identified to date that is preferentially expressed in developing embryos (Rounsley et al., Plant Cell 7:1259-1269, 1995). A comparison of the predicted amino acid sequence of AGL15 to predicted amino acid sequences encoded by other *Arabidopsis* MADS domain genes showed a high percentage of amino acid identity in the 56-amino acid MADS domain, a lower percentage of amino acid identity in the K domain, and a divergence of amino acid sequences in the C-terminal region.

Generation of AGL-15-Specific Antibodies

AGL15-specific antigen was obtained as follows. Nucleotide sequences downstream of the MADS domain of the *B. napus* AGL15-1 gene were amplified from the *B. napus* transition stage embryo cDNA library. The primers used in the amplification reaction were AGL15-1-specific oligonucleotides that were flanked by *Nco*I and *Bam*HI restriction sites, and which incorporated a termination codon. The PCR product, which corresponded to amino acid residues 62 to 258 of SEQ ID NO:3, was ligated to a linearized expression vector pET-15b (Novagen, Madison, WI) with compatible ends.

Overexpression of truncated *B. napus* AGL15-1 was accomplished by transformation of the expression host BL21(DE3) and induction with 1mM isopropyl  $\beta$ -D-thiogalactopyranoside (X-Gal) (Perry and Keegstra, Plant Cell 6:93-105, 1994). The polypeptide was recovered from isolated inclusion bodies by solubilization for five minutes at room temperature in a solution containing 8M urea and 10 mM  $\beta$ -mercaptoethanol in a 50 mM Tris-HCl, 5mM MgCl<sub>2</sub> buffer, pH 7.6. The solubilized protein

was further purified by electrophoresis on two successive preparative Pro-Sieve agarose gels (FMC, Rockland, ME). A protein band corresponding to truncated AGL15-1 was excised from the gel and used to immunize rabbits at the University of Wisconsin-Madison Medical School Animal Care Unit.

Blot-affinity purification (Tang, Methods in Cell Biology, 37:95-104, 1993) was used to purify antibodies that recognized truncated AGL15-1 for use in protein gel blot analyses, described below. Antibodies to be used in immunohistochemistry studies were prepared as follows. Immune and preimmune sera were preadsorbed to remove serum components that bind nonspecifically to fixed plant tissues (Jack et al., Cell 76:703-716, 1994). Pieces (approximately 4 mm<sup>2</sup>) of fully expanded Brassica leaves in which AGL15 is not expressed were fixed for one hour under vacuum with 4% (w/v) freshly prepared paraformaldehyde and 0.02% (v/v) Triton X-100 in 50 mM potassium phosphate buffer, pH 7. The leaf pieces were washed for several hours in a large volume, with multiple changes, of PBST buffer (237 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, 0.1 % Tween 20, pH 7.3). A solution consisting of 10% (v/v) preimmune or immune serum, 0.05% (w/v) BSA fraction V in 0.9X PBST was added to the fixed leaf pieces (approximately 5 ml of solution per gram of leaf tissue) and incubated overnight at 4° C with gentle agitation. The preadsorbed serum was removed by aspiration, and sodium azide was added to make the serum 0.05% (w/v) sodium azide. The serum was stored at 4° C. Serum prepared in this manner could be used for several months.

Protein extracts of developing plant embryos for immunoblot analysis were prepared as described in Heck, et al. (Heck, et al., Plant Cell 7:1271-1282, 1995). Plant tissue sections were prepared and immunohistochemistry performed as described in Perry, et al. (Perry, et al., Plant Cell 8:1977-1989, 1996).

Several lines of evidence indicate that the AGL15 antiserum is specific for AGL15. Gel blot analysis demonstrated that the AGL15 antiserum does not recognize AGL2, which is the only other MADS domain protein reported to be



expressed during embryogenesis in Arabidopsis (Flanagan and Ma, Plant Mol. Biol. 26:581-595, 1994). Immunohistochemical studies employing Brassica embryos demonstrated that AGL15 antiserum exhibits nuclear staining in developing embryos.

5 However, antiserum depleted of AGL15-specific antibodies by preadsorption with overexpressed AGL15 did not exhibit nuclear staining (Perry and Fernandez, unpublished results). To determine whether the antibodies recognize and bind other MADS domain proteins, sections of young floral buds were incubated  
10 with antiserum. The antibodies did not label nuclei in developing floral organs, a developmental context in which many different MADS domain family members are expressed in Arabidopsis.

Conservation of AGL15 Structural Elements within Angiosperms

15 If the AGL15 gene product plays an important role in embryo development, it is reasonable to expect that a related protein performs similar functions in embryos of many different groups of flowering plants. This hypothesis was tested using the AGL15-specific antibodies in combination with immunoblots  
20 of soluble protein extracts from numerous groups of flowering plants, and immunohistochemistry, using sections of plant embryos and young seeds. In immunoblot analysis, the AGL15 antibodies were found to bind to one, or at most two, protein band(s) from all tested plant embryos. Immunohistochemistry  
25 using sections from developing embryos from a variety of plant showed that the AGL15-specific antibody bound to embryo sections from all tested plant groups, and that the staining was localized to the nuclei. These results are summarized in Table 1.

TABLE 1  
Detection and Localization of AGL15  
Proteins in Flowering Plants

	<u>Plant</u>	<u>Tissue</u>
5	<i>Brassica napus</i> (oilseed rape)	embryo/endosperm (seed) inflorescence, abscission zone, developing pollen, somatic embryo young seedling
	<i>Arabidopsis thaliana</i>	embryo/endosperm (seed) inflorescence, young seedling
	Broccoli	inflorescence
	Cauliflower	inflorescence
10	Cleome	inflorescence
	Polanisia	inflorescence
	Papaya	embryos
	Pepper	seed
	Zea mays (maize)	embryo/endosperm (seed)
15	Potato	abscission zone
	Tomato	abscission zone
	Wheat	wheat germ (embryos)
	Dandelion	embryos (seed)
	Alfalfa	leaves and somatic embryos
20	Rice	embryos
	Chicory	leaves and somatic embryos vegetative shoot in culture

The temporal and spatial patterns of expression of AGL15 are consistent with it being a factor in embryo specification.

25 AGL15 mRNA is present throughout embryo development and maturation, and is present in all cells of the embryo. This pattern of expression suggests that AGL15 may have a global regulatory function, such as the promotion of embryo-specific programs or the inhibition of postgermination programs (Heck et

30 al. Plant Cell 7:1271-1282, 1995). The ubiquitousness and the high degree of conservation of the AGL15 gene among plants suggest that it has an essential function in plant development. To facilitate research into the role of AGL15 in plant development, transgenic plants in which AGL15 was overexpressed

35 were created.

Generation of Genetic Constructs and Transformation of Plants

Two constructs containing an AGL15 gene operably linked to a promoter functional in plants were created using the transformation vector pBI121 (Clontech). An AGL15 protein-  
5 encoding DNA sequence (SEQ ID NO:1) was placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter. This was accomplished by replacing the GUS gene of pBI121 with the Arabidopsis AGL15 cDNA sequence (SEQ ID NO:1), which  
10 contains an 807-bp ORF, as well as 18 bp of the 5' untranslated region (UTR) and 245 bp of the 3' UTR. The construct was designated p35S-AGL15 (DF164) (Fig 1A). A second construct, designated p35S-AGL15+ (DF121), was made by replacing a BsmI-NsiI  
15 fragment within the ORF of the Arabidopsis AGL15 cDNA insert in the DF164 construct with the first three introns of the genomic AGL15 gene (Fig. 1B). This construct was made with the expectation that it would afford higher levels of AGL15 expression, because introns are sometimes necessary to achieve high levels of gene expression.

Constructs were transformed into Arabidopsis with  
20 *Agrobacterium* strain GV3101 using the vacuum infiltration protocol (Bechtold, et al., Comptes Rendus de l'Academie des Sciences Serie III Sciences de la Vie 360:1194-1199, 1993) and modifications introduced by A. Bent to simplify plant handling. Transformants (T1 generation) were selected on GM plates  
25 supplemented with 75 µg/ml kanamycin prior to transfer to soil. The number of transgenic loci within each line was determined by segregation of kanamycin resistance (using 50 µg/ml kanamycin) in T2 progeny.

The relative levels of ectopic expression were determined  
30 by preparing soluble protein extracts from leaves, which normally do not accumulate AGL15, and subjecting the protein extracts to immunoblot analysis. Transformation of plants with the DF164 construct yielded transgenic plants in which AGL15 was constitutively expressed at low to intermediate levels.  
35 Transformation of plants with the DF121 construct, which contains three introns, yielded transformants in which AGL15

was constitutively expressed at intermediate to high levels.

#### Characterization of Transgenic Plants

In initial experiments, transformation of *Arabidopsis* plants with DF164 yielded 48 lines carrying the construct. Of these 48 lines, only one line showed an obvious phenotypic distinction in the T1 generation. The same phenotypic alteration was seen in the T2 generation in several more lines, presumably because the DF164 copy number increased after the T1 plants selfed. The phenotypically distinct plants were found to have an intermediate level of overexpression of the AGL15 gene. Several other lines of DF164 transformants that exhibit the phenotype and intermediate levels of AGL15 expression have been obtained in subsequent trials; characterization of these lines is currently underway. Transformation of *Arabidopsis* with DF121 yielded 38 lines, of which 17 demonstrated obvious phenotypes that corresponded to intermediate or high levels of overexpression in the T1 generation.

A total of 20 lines exhibited altered phenotypes associated with AGL15 overexpression. These phenotypes fell into two classes, which corresponded to different levels of overexpression, as assessed by immunoblot analysis of leaf soluble protein samples. Class 1 plants, in which AGL15 was overexpressed at intermediate levels, showed a variety of effects. The effects observed include: 1) delayed silique (fruit) maturation; 2) increased numbers of flowers and fruits; 3) delayed floral organ senescence/abscission; and 4) delayed senescence of cut flowers and inflorescences.

Class 2 plants, in which AGL15 was overexpressed at high levels, showed a variety of severe (abnormal) phenotypes, as well as many of the features characteristic of the Class 1 plants. Both the leaves and cotyledons of Class 2 plants appeared to have expansion problems, and produced "cupped" organs with upturned margins. The flowers were semi- or completely sterile and showed features that suggest that high levels of AGL15 interfere with the function of other MADS domain regulatory factors. Floral petals were green. In the two lines that demonstrated the highest level of

overexpression, up to 30% of the flowers had 4-5, rather than 2, carpels and they contained another inflorescence within the fused carpels. The two fused carpels are also carried on an elongated internode. Seeds produced by outcrossing strong overexpressors were abnormally shaped but contained normal levels of storage protein. However, they appeared to be dessication intolerant and did not germinate when they were left on the plant until the siliques were fully dry.

#### Effects of Overexpression of AGL15 on Fruit Maturation

Fruit maturation in transgenic *Arabidopsis* plants that contained a single copy of DF164 and that exhibited intermediate overexpression of AGL15 was compared with fruit maturation in untransformed *Arabidopsis* controls. Transgenic *Arabidopsis* plants that exhibited high levels of AGL15 overexpression were self-sterile and did not produce fruit. In assessing the effects of AGL15 on fruit maturation, the "time to maturity" was defined as the number of days from pollination to full maturity. Fruits were considered to have reached "full maturity" when they were completely brown. The time to maturity was approximately 50% longer in transgenic plants than in untransformed controls (Table 2).

TABLE 2

#### Effects of AGL15 Overexpression on Fruit Maturation in *Arabidopsis*

25	Time (days) from pollination to full maturity		
	<u>Genotype</u>	<u>Experiment 1</u>	<u>Experiment 2</u>
	wildtype	17.25 $\pm$ 0.9 (N=59)	18.4 $\pm$ 0.6 (N=29)
30	transgenic	24.6 $\pm$ 0.7 (N=17)	26.2 $\pm$ 0.8 (N=44)

#### Effect of AGL15 Overexpression on Fruit Production

Transgenic *Arabidopsis* plants containing a single copy of the DF164 construct were grown adjacent to untransformed *Arabidopsis* control plants until the plants had matured and

dried fully. The number of siliques (fruit) produced by each plant was determined. Only those siliques that showed good seed fill and that were produced in the initial phase of inflorescence growth (before the point of global arrest, when the meristems "pause") were counted as "fruit". A comparison of the number of siliques produced showed that the transgenic plants produced approximately 50% more fruit than the untransformed controls (Table 3).

TABLE 3

Effects of AGL15 Overexpression  
on Fruit Production

Genotype	No. of siliques per plant
wildtype	381 $\pm$ 64 (N=5)
transgenic	750 $\pm$ 149 (N=5)

Effect of AGL15 Overexpression on Floral Organ Abscission and Senescence

In untransformed Arabidopsis plants, petals and sepals undergo abscission from two to three days after pollination. In transgenic plants in which AGL15 is overexpressed at intermediate levels, petals and sepals remain attached for from 1.5 to 2 weeks following pollination. The floral organs remain turgid and show no sign of senescence during this period. Transgenic plants in which AGL15 was expressed at high levels showed delayed abscission and senescence that was more dramatic than plants with intermediate levels of expression. However, the flowers of these plants were not normal, in that the floral petals were green.

Effects of Overexpression of AGL15 on Cut Flower Longevity

The effects of AGL15 overexpression on the longevity of cut flowers was assessed as follows. Flowers and/or inflorescences were removed from transgenic and untransformed plants and placed on filter paper moistened with distilled water, and the filter paper transferred to a dish that was then

sealed to maintain high humidity. The sealed dishes containing the cut flowers were incubated under ambient temperature and light conditions. Flowers from untransformed plants turned brown within a few days. Flowers from transgenic plants lived  
5 up to 2.5 weeks without showing signs of senescence, in that the sepals and stems remained green and the petals remained turgid. As long as high humidity was maintained, the cut flowers exhibited no sign of wilting. However, growth of contaminating mold necessitated termination of the experiments  
10 at around three weeks, prior to any sign of floral wilting. The experiment was repeated several times, with 10 to 20 flowers in each experimental set. The effect was even more pronounced in plants overexpressing AGL15 at high levels, in that after 2.5 to 3 weeks, even the oldest flowers at the base  
15 of the cut inflorescence had the appearance of newly opened flowers. It is speculated that the more pronounced effect observed in plants in which AGL15 is expressed at high levels is related to the reduced fertility that these plants exhibit.

Because research in the area of flower senescence and  
20 abscission has focused on the manipulation of ethylene levels, the response of the transgenic plants to ethylene was assessed using the cut flower assay. When transgenic plants in which AGL15 is overexpressed and which exhibited delayed floral abscission were exposed to ethylene, their petals fell off the  
25 plant. Arabidopsis mutant *etr-1* plants, which do not lose their flower petals upon exposure to ethylene, were included in the cut flower assay. These plants retain petals and sepals for a few days longer than wild type Arabidopsis plants, but not as long as the transgenic plants overexpressing AGL15.  
30 These results suggest that AGL15 may affect some aspect of the senescence/abscission process that is ethylene-independent.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Fernandez, Donna E.  
Heck, Gregory R.
- 5 (ii) TITLE OF INVENTION: EXPRESSION OF AGL15 SEQUENCE IN  
TRANSGENIC PLANTS
- (iii) NUMBER OF SEQUENCES: 7
- (iv) CORRESPONDENCE ADDRESS:
- 10 (A) ADDRESSEE: Quarles & Brady  
(B) STREET: 1 South Pinckney Street  
(C) CITY: Madison  
(D) STATE: WI  
(E) COUNTRY: US  
15 (F) ZIP: 53701-2113
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
20 (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER: US  
(B) FILING DATE:  
(C) CLASSIFICATION:
- 25 (viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Seay, Nicholas J.  
(B) REGISTRATION NUMBER: 27,386  
(C) REFERENCE/DOCKET NUMBER: 960296.94193
- (ix) TELECOMMUNICATION INFORMATION:
- 30 (A) TELEPHONE: (608) 251-5000  
(B) TELEFAX: 608-251-9166

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
- 35 (A) LENGTH: 1070 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
- |    |   |     |
|----|---|-----|
| 40 | GTTCAATTTT GGGGAAAAT GGGTCGTGGA AAAATCGAGA TAAAGAGGAT CGAGAATGCG  | 60  |
|    | AATAGCAGAC AAGTCACTTT TTCCAAGAGG CGTCTGGGT TACTTAAGAA AGCTCGTGAG  | 120 |
|    | CTCTCTGTTT TTTGTGATGC TGAAGTTGCT GTCATCGTCT TCTCTAAGTC TGGCAAGCTC | 180 |
|    | TTTCAGTACT CCACTACTGG AATGAAGCAA ACACTTTCCA GATACGGTAA TCACCAGAGT | 240 |
|    | TCTTCAGCTT CTAAAGCAGA GGAGGATTGT GCAGAGGTGG ATATTTTAAA GGATCAACTT | 300 |
| 45 | TCAAAGCTTC AAGAGAAACA TTTACAACGT CAGGGCAAGG GCTTGAATCC TCTGACCTTT | 360 |
|    | AAAGAGCTGC AAAGCCTTGA GCAGCAACTA TATCATGCAT TGATTACTGT CAGAGAGCGA | 420 |



	AAGGAACGAT TGCTGACTAA CCAACTTGAA GAATCACGCC TCAAGGAACA ACGAGCAGAG	480
	TTGGAAAACG AGACCTTGCG TAGACAGGTT CAAGAACTGA GGAGCTTTCT CCCGTCGTTT	540
	ACCCACTATG TTCCATCCTA CATCAAATGC TTTGCTATAG ATCCAAAGAA CGCTCTCATA	600
	AACCACGACA GTAAATGCAG CCTCCAGAAC ACCGATTCAG ACACAACTTT GCAATTAGGG	660
5	TTGCCGGGAG AGGCACATGA TAGAAGGACG AATGAAGGAG AAAGAGAGAG CCCGTCGAAGC	720
	GATTCACTGA CAACAAACAC GAGCAGCGAA ACTGCAGAAA GAGGGGATCA GTCTAGTTTA	780
	GCAAAATCTC CACCTGAAGC CAAAAGACAA AGGTTCTCTG TTTAGTCCTA GAAAAGTATG	840
	GGAGAAGGCT ACTAATGTTT CCTCTTTAGC AGTATCCGAT TGTTTTAAAA GTAATTTTAG	900
	AGGGATACTT GCAAAAAGAA GAGAAGATTC AGTTATCTAA TCTCTGCACC AACTCTCTTT	960
10	GTCTCTCTTC TTTTGATTAT TTCTCGACTG TCTCTCTAT AAAAAAGATA TGCCTAGCTG	1020
	AGAGTTTGAA ATCCATAATC TTTACAAGGC ACAGAGTTAT TTGACAAAAA	1070
	(2) INFORMATION FOR SEQ ID NO:2:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 795 base pairs	
15	(B) TYPE: nucleic acid	
	(C) STRANDEDNESS: double	
	(D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: cDNA	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:	
20	ATGGGTCGTG GAAAAATTGA GATAAAGAGG ATCGAGAATG CGAATAGCAG GCAAGTTACC	60
	TTCTCCAAGA GCGGTGCTGG TTTGCTCAAG AAAGCTCATG AGCTCTCTGT TCTTTGTGAC	120
	GCTGAGGTG CCGTCATTGT CTTCTCCAAG TCTGGCAAGC TCTTCGAGTT CTCAGTACT	180
	AGCATGAAGA AAACACTTTT GAGATACGGA AATTATCAGA TCTCTTCAGA TGTTCCTGGG	240
	ATTAAGTGA AAACAGAGAA CCAGGAGGAG TGTACAGAGG TGGACCTTTT AAAGGATGAG	300
25	ATCTCAATGC TTCAAGAGAA ACATTTACAC ATGCAGGGTA AGCCCTTGAA CCTTCTGAGC	360
	TTGAAAGAGC TGCAACACCT TGAGAAGCAA CTAAATTTCT CATTGATATC TGTGAGAGAG	420
	CGAAAGGAAC TATTGTTGAC TAAACAACTT GAAGAGTCAC GGCTTAAGGA ACAGAGAGCA	480
	GAGCTGGAAA ACGAGACCTT ACGTAGACAG GTTCAAGAAC TAAGGAGTTT TCTCCCGTCG	540
	ATCAACCAAC ACTATGCTCC ATCCTACATC AGATGCTTCG CTATAGATCC TAAGAACTCA	600
30	CTCTTAAGCA ACACTTGCTT GGGCGACATT AACTGCAGCC TCCAGAACAC CAACTCAGAC	660
	ACAACTTTGC AACTAGGTTT GCCGGGAGAA GCACATGATA CAAGGAAGAA CGAAGGAGAC	720
	AGAGAGAGCC CATCAAGTGA TTCTGTGACA ACGAGCACAA CCAGAGCAAC TGCACAAAGG	780
	ATCAGTCTAG TTTAG	795

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 264 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS:  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

5 Met Gly Arg Gly Lys Ile Glu Ile Lys Arg Ile Glu Asn Ala Asn Ser  
 1 5 10 15  
 10 Arg Gln Val Thr Phe Ser Lys Arg Arg Ala Gly Leu Leu Lys Lys Ala  
 20 25 30  
 His Glu Leu Ser Val Leu Cys Asp Ala Glu Val Ala Val Ile Gly Phe  
 35 40 45  
 15 Ser Lys Ser Gly Lys Leu Phe Glu Phe Ser Ser Thr Ser Met Lys Lys  
 50 55 60  
 Thr Leu Leu Arg Tyr Gly Asn Tyr Gln Ile Ser Ser Asp Val Pro Gly  
 65 70 75 80  
 20 Ile Asn Cys Lys Thr Glu Asn Gln Glu Glu Cys Thr Glu Val Asp Leu  
 85 90 95  
 Leu Lys Asp Glu Ile Ser Met Leu Gln Glu Lys His Leu His Met Gln  
 100 105 110  
 Gly Lys Pro Leu Asn Leu Leu Ser Leu Lys Glu Leu Gln His Leu Glu  
 115 120 125  
 25 Lys Gln Leu Asn Phe Ser Leu Ile Ser Val Arg Glu Arg Lys Glu Leu  
 130 135 140  
 Leu Leu Thr Lys Gln Leu Glu Glu Ser Arg Leu Lys Glu Gln Arg Ala  
 145 150 155 160  
 30 Glu Leu Glu Asn Glu Thr Leu Arg Arg Gln Val Gln Glu Leu Arg Ser  
 165 170 175  
 Phe Leu Pro Ser Ile Asn Gln His Tyr Ala Pro Ser Tyr Ile Arg Cys  
 180 185 190  
 Phe Ala Ile Asp Pro Lys Asn Ser Leu Leu Ser Asn Thr Cys Leu Gly  
 195 200 205  
 35 Asp Ile Asn Cys Ser Leu Gln Asn Thr Asn Ser Asp Thr Thr Leu Gln  
 210 215 220  
 Leu Gly Leu Pro Gly Glu Ala His Asp Thr Arg Lys Asn Glu Gly Asp  
 225 230 235 240  
 40 Arg Glu Ser Pro Ser Ser Asp Ser Val Thr Thr Ser Thr Thr Arg Ala  
 245 250 255  
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 260

## (2) INFORMATION FOR SEQ ID NO:4:

## (1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2679 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

## (11) MOLECULE TYPE: DNA (genomic)

## (x1) SEQUENCE DESCRIPTION: SEQ ID NO:4:

	AAGCTTTGGT TGTACGGGTC AAAGTATTCG TTCTGGGGTG GAGTTGGAGA AGCCTTCAGA	60
10	GCCAGTTTAG TAAGGGTTCT TCGAGGGAGG TCTGTATAGA AAGTAGCAAG CAGAACATGT	120
	TGGCCTTGTC TAATGTAGAT AGTTGTAATC AGTGGTGCTA CAATGTTGTC TGATGGAATT	180
	AAATTTCCTAA ATGGTCAAAA TGAAAACGTT GAAGAAAAAA AAAACAATTG TGATATGATG	240
	ATCTTCCTAC TTATATCATA TGATCTGCAC GGATTAGAAT TGTGTTTGAG AGTATATGAT	300
	CTGGATTCTT TGTTCTGTTA AATTCTGAG TCAATTCAAA ATCATATTTT CCTTCGTTGA	360
15	TAAAATTATG TGTTCACTTT TTCTAGCTCT GTACAAAAAT CAATCAACTG ATTTGTTATT	420
	TGTATAGTTA TTTGTTTTTT ACCAAGTCTT GCTCTGATT TTTTTTTTA GTCTTGCTCT	480
	GATTTATACC ATCAACATCA AGTACATTTT TTCGTGGTCA AACATCAAGT ACAATTTTTA	540
	TATTAGCGTA AACAAATATA AAGAAATATT GTTTTGTCTG GCAGAATAAA AGAAATATAA	600
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20	CAACTGTTAT GGCAACAAAA GGAACCGTGG GTCCCAGAGG AACTGGCAAA CCCTCTAAAT	720
	GTGGCAAAAA GGTGTCATGC AAATACTCTA AAAGAGAGAG AGAGAGGAGC ACGCAAAACA	780
	GTGCTCATGC AAACACAAAC ACAGTTAAGT TTCTTTGTAG TTTGTACTAA TCTCTCTTTT	840
	TTATATATAT ATTACATCCA AATATAGCAA ATCTTTGTGT CTTCCTTTTA TAGATTGTAA	900
	CCCCAAAAAG GAGTTTCAAT AGGGAAGAAG AGAGATTGAA ACTCCTTTTC TTTCTTCATC	960
25	TTCTTTTTTC TCTTCTGTGC TTGAAGATGG GTCGTGGAAA AATTGAGATA AAGAGGATCG	1020
	AGAATGCGAA TAGCAGGCAA GTTACCTTCT CCAAGAGGCG TGCTGGTTTG CTCAAGAAAG	1080
	CTCATGAGCT CTCTGTTCTT TGTGACGCTG AGGTGCGCGT CATTGTCTTC TCCAAGTCTG	1140
	GCAAGCTCTT CGAGTTCTCA AGTACTAGGT GGTAATTAAT CAATCATTTT CTGATTCCA	1200
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30	GTTAACCTGG TTCTTGCAATG TTTGTTTAGA TTCATTAGTC CTAATTAATC TCACATTGTC	1320
	TTCTTAGATC TAATTCTCA TTTGGTTTTT AGCATGAAGA AAACACTTTT GAGATACGGA	1380
	AATTATCAGA TCTCTTCAGA TGTTCCTGGG ATTAAGTGA AAACAGAGGT TAGAACTCA	1440
	TGTGGTTTTT GCCTAGACTC AACTCAAGTG TTTTGAAGTG TTTGTCTCG ATGCATCAAA	1500
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35	TGCTTCAAGA GAAACATTG TATGGAACCC AATCCTAAT TATATTATTT TTTCCCCACA	1620
	CCATCCACCA CTTTGTGTG TCTTATATGG TTTGTCTTG TGTGTGTTG TAGACACATG	1680

	CAGGGTAAGC CCTTGAACCT TCTGAGCTTG AAAGAGCTGC AACACCTTGA GAAGCAACTA	1740
	AATTTCTCAT TGATATCTGT GAGAGAGCGA AAGGTAAAAA ACTAGTAATA TCACTCTTCC	1800
	CCATTTCTTT TCTCATTAAA AACATATTTG CATTITTTCTG AATAAAAGTT TATGTGATTT	1860
	CAGGAACTAT TGTGACTAA ACAACTTGAA GAGTCACGGC TTAAGGTAAC TCTTGAGTGA	1920
5	TATGAAACAC TTGATTTTTT CTGATTAGCT TCTAAGCATG CAAGATTATG TGATCACATG	1980
	ATTCTGATGA ACCGTTTTAA AAATGTATGT CCCCTCTTTA CTGCCTATTG TATCCTTTGA	2040
	GAGGGTTCAT GTTGTAGCTA GCTATCTTAA CTGAGTATGA TGCAATAGTT GATCATCTAG	2100
	AGCATTGAAA CTCTGCAGGA ACAGAGAGCA GAGCTGGAAC ACGAGACCTT ACGTAGACAG	2160
	GTGACGAAAC CATTCTTATA ATTTGTGTTG TATCATCTCT TATCACCAG TCTTCTTTTT	2220
10	ACTACTTCTA ATCAGCTTCT CTTGAAAAATA GGTTCAGAA CTAAGGAGTT TTCTCCCGTC	2280
	GATCAACCAA CACTATGCTC CATCTACAT CAGATGCTTC GCTATAGATC CTAAGAAGTC	2340
	ACTCTTAAGC AACACTTGCT TGGGCGACAT TAACTGCAGC CTCCAGAACA CCAACTCAGA	2400
	CACAACTTTG CAACTAGGGT ATGTGCTCTT TTAACCTTTT TTGCTACCAT TGGTTGCACT	2460
	ATAGTTAGCC AAAAGTACTC TTCTAGTATA CATATGCATT AACACTATTG GACTTATTAA	2520
15	TTCTCATATG TGTGTTTTTC TTGAAGGTTG CCGGGAGAAG CACATGATAC AAGGAAGAAC	2580
	GAAGGAGACA GAGAGAGCCC ATCAAGTGAT TCTGTGACAA CGAGCACAAC CAGAGCAACT	2640
	GCACAAAGGA TCAGTCTAGT TTAGAAACTA TTTTCATCTG	2679

## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 951 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

	GAGATAAAGA GGATCGAGAA TGCGAATAGC AGACAAGTTA CTTTCTCCAA GAGGCGTGCT	60
	GGTTTGCTCA AGAAGGCTCA TGAGCTCTCT GTTCTTTGCG ACTCTGAGGT TGCCGTCATC	120
	GTCTTCTCCA AGTCCGGCAA GCTCTTCGAG TTCTCAAGTA CTGGCATGAA GCGAACCGTT	180
	TTGAGATACG AGAACTACCA ACGTTCTTCA GATGCTCCTC TGATTAAATA TAAACCAAGAG	240
30	AACCAGGAGG AGGATTGTAC AGAGGTGGAC TTTTAAAGA ATGAGATCTC AAAGCTTCAA	300
	GAGAAACATT TACAAATGCA AGGTAAGGGC TTGAATGCTC TGTGCTTGAA AGAGCTGCAA	360
	CACCTTGAAC AGCAACTAAA TGTCTCGTTG ATATCTGTGA GAGAGCGAAA AGAACTATTG	420
	TTGACTAAAC AAATTGAAGA ATCAGTATC AGGGAACAGA GAGCAGAGCT GGAAAACGAG	480
	ACCTTACGTA GACAGGTTCA AGAACTAGA AATTTCTCC CGTCCATCAA CCAAACTAT	540
35	GTCCATCCT ACATCATATG CTTGCTATA GATCCCAAGA ACTCCCCCGT GAACAACTCT	600
	GGCTTGACG AACTAATTA CAGTCTCCAG AAGACCAATT CAGACACAAC ATTGCAGTTG	660

GGGTTGCCGG GAGAAGCACA GGCTAGAAGG AGGAGTGAAG CAAATGAGA GAGCCCATCA 720  
 AGTGATTTCAG TAACAACGAG CACCACCAAA GCAACTCCAC AAAGGATCAA TCTAGTTTAG 780  
 CACCTGAAAA CAAAAGCAAA TGGTTCTCTG CTTAGCCACA TAGAAATATG GGAATGAGGC 840  
 ACATGATGTT TTCCTCTGTA GCAAGTATCA CATTATTTCA AAACCAATGT TAGAAGAGAT 900  
 5 GAATCCGATG TATCTCATCT CACATTCTAG TCTAACTCTA ACCCCACTCT T 951

## (2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 2437 base pairs  
 (B) TYPE: nucleic acid  
 10 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATCAACAATG CTAGTTGTTG CATTTTATTC TTGGGGTACT TTGAAATTGT TTCTAATTGT 60  
 15 TGCTCTGAAC TTTTITATTT TATGTCGGTC AACATTGTTG CTCTGATTTA TGTCTTACAA 120  
 CAACATTAAG GAGAAAATAC ACTAGTACTA ATAAATCTAA TTTTAAAGAG AAGGAAAAAA 180  
 GAGGAAAGAA AAAACAACCT TTAGGAAGAA AAGGGAAAGT AGGACCCAGA AGAACTGACA 240  
 AAATCCTCCA AATGTGGCAA AAAGGTATCA TGCAAAAAAC CCTAAAATTG AAAAAAGAGA 300  
 GCACGCAAAA CAGTGGCCAT GCAACACACA ATATTCATTA CCGAGTTTTT ACCTTTCTTT 360  
 20 CTTTTTCTA TAAAAAATAA AATATTCCAT CCAAATTTAG CAATCTTTTG TGTTCCTATT 420  
 AATAGATTCC CAAAAGCAC TTCTAAACCC ATTTTGAAT ACATTGAACC TTCTCTCTTC 480  
 TTCTTCTTCC TTCTACCTTC TTCTCTCTGT TCAATTTTGG GGGAAAATGG GTCGTGGAAA 540  
 AATCGAGATA AAGAGGATCG AGAATGCGAA TAGCAGACAA GTCACTTTTT CCAAGAGGCG 600  
 TTCTGGGTGA CTTAAGAAAG CTCGTGAGCT CTCTGTTCTT TGTGATGCTG AAGTTGCTGT 660  
 25 CATCGTCTTC TCTAAGTCTG GCAAGCTCTT CGAGTACTCC AGTACTGGGT AACACTTATT 720  
 TCTTTTIGAT TCAATTTTGG TTTTGCATGT CTGTCTTGT TGTGATTAGA ATCGATTTCG 780  
 GGAAGTGATA TTGATTTTGG TTTTGCATG TTTGTTAAGA TTAAAAGTTT TCTGATTGAG 840  
 CTGAAGAGAG TCCTAATTTT GAATTCTCAT TTGATTTTAG AATGAAGCAA ACACTTTCCA 900  
 GATACGGTAA TCACCAGAGT TCTTCAGCTT CTAAGCAGA GGTGAGAATC ATTCATTCTT 960  
 30 GTCTCATATA TCTTGAAATT GTTTTGTGTA AAATCTGATT GCTGTTTAGA ACCTCCAGGA 1020  
 GGATTGTGCA GAGGTGGATA TTTTAAAGGA TCAACTTTCA AAGCTTCAAG AGAAACATTT 1080  
 GTATGGAAAC TAAATAAATC TCACTATGCT TGTTCATTAC TTTATTCTTC TCTACTTTGT 1140  
 GTTTGTTTAT ATTGTTTGGC TTTGTGTGTT CTGTTCTGTT GTAGACAACT GCAGGGCAAG 1200  
 GGCTTGAATC CTCTGACCTT TAAAGAGCTG CAAAGCCTTG AGCAGCAACT ATATCATGCA 1260  
 35 TTGATTACTG TCAGAGAGCG AAAGSTAAT AGTAATATCA CTCTTCCATG ATCATTCTTC 1320  
 TTTGCATTGT CCTGATTATG GTTATCTGAT TTCAGGAACG ATTGCTGACT AACCACCTTG 1380

AAGAATCACG CCTCAAGGTA AACACTAGCT TTTCTCTCT AGCTTCCAAA TGTAAGCTTA 1440  
 TGTGTAATCA CATGATTCTG AACCTTGTTA AAACCAAGTG CTATCCTTTG ACAAGCTCAT 1500  
 GCTCTAACTA GCTAGTGTGC AGTTTATTG TCTTAAGACT CCTATATAAC TAGGTACAGA 1560  
 GTACAAAAGT ATAATTTCTT GATTAGCCAT ATATATACTT TGCAGGAACA ACGAGCAGAG 1620  
 5 TTGGAACACG AGACCTTGGC TAGACAGGTT CTTATTATTT TTGTTGAATC ATCTCCTAAT 1680  
 GAACGCTTCT TCCTCTGACT TGTAAATTACT TGTGAAACA GGTTCAGAA CTGAGGAGCT 1740  
 TTCTCCCGTC GTTCAACCCAC TATGTTCCAT CCTACATCAA ATGCTTTGCT ATAGATCCAA 1800  
 AGAACGCTCT CATAAACCAC GACAGTAAAT GCAGCCTCCA GAACACCGAT TCAGACACAA 1860  
 CTTTGCAATT AGGGTATTGC TCTTTTAACT CTATTTGCTG TCATTGGTTG CATTATGGGA 1920  
 10 AAGCTGATTT AAGATAAATA TAAGTCTTTT TCCTCCTCTG TTAGTTATGC ATATGCCTTA 1980  
 ACACTCACTA ACTGGTGTTA TAAATTCCTT ACTACTTGTG TTTTCTCCAA GGTGCGGG 2040  
 AGAGGCACAT GATAGAAGGA CGAATGAAGG AGAAGAGAG AGCCCGTCAA GCGATTCACT 2100  
 GACAACAAAC ACGAGCAGCG AAACGTCAGA AAGAGGGGAT CAGTCTAGTT TAGCAAATTC 2160  
 TCCACCTGAA GCCAAAAGAC AAAGGTTCTC TGTTTAGTCC TAGAAAAGTA TGGGAGAAGG 2220  
 15 CTACTAATGT TTCCTCTTTA GCAGTATCCG ATTGTTTTAA AAGTAATTTT AGAGGGATAC 2280  
 TTGCAAAAAG AAGAGAAGAT TCAATTATCT AATCTCTGCA CCAACTCTCT TTGTCCTTCT 2340  
 TCTTTTGATT ATTTCTGAC TGTCTCTCCT ATAAAAAGA TATGCCTAGC TGAGAGTTTG 2400  
 AAATCCATAA TCTTTACAAG GCACAGAGTT ATTTGAC 2437

## (2) INFORMATION FOR SEQ ID NO:7:

20 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 268 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS:  
 (D) TOPOLOGY: linear  
 25 (ii) MOLECULE TYPE: protein  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:  
 Met Gly Arg Gly Lys Ile Glu Ile Lys Arg Ile Glu Asn Ala Asn Ser  
 1 5 10 15  
 Arg Gln Val Thr Phe Ser Lys Arg Arg Ser Gly Leu Leu Lys Lys Ala  
 20 25 30  
 Arg Glu Leu Ser Val Leu Cys Asp Ala Glu Val Ala Val Ile Val Phe  
 35 40 45  
 Ser Lys Ser Gly Lys Leu Phe Glu Tyr Ser Ser Thr Gly Met Lys Gln  
 50 55 60  
 35 Thr Leu Ser Arg Tyr Gly Asn His Gln Ser Ser Ser Ala Ser Lys Ala  
 65 70 75 80  
 Glu Glu Asp Cys Ala Glu Val Asp Ile Leu Lys Asp Gln Leu Ser Lys  
 85 90 95

Leu Gln Glu Lys His Leu Gln Leu Gln Gly Lys Gly Leu Asn Pro Leu  
 100 105 110  
 Thr Phe Lys Glu Leu Gln Ser Leu Glu Gln Gln Leu Tyr His Ala Leu  
 115 120 125  
 5 Ile Thr Val Arg Glu Arg Lys Glu Arg Leu Leu Thr Asn Gln Leu Glu  
 130 135 140  
 Glu Ser Arg Leu Lys Glu Gln Arg Ala Glu Leu Glu Asn Glu Thr Leu  
 145 150 155 160  
 10 Arg Arg Gln Val Gln Glu Leu Arg Ser Phe Leu Pro Ser Phe Thr His  
 165 170 175  
 Tyr Val Pro Ser Tyr Ile Lys Cys Phe Ala Ile Asp Pro Lys Asn Ala  
 180 185 190  
 Leu Ile Asn His Asp Ser Lys Cys Ser Leu Gln Asn Thr Asp Ser Asp  
 195 200 205  
 15 Thr Thr Leu Gln Leu Gly Leu Pro Gly Glu Ala His Asp Arg Arg Thr  
 210 215 220  
 Asn Glu Gly Glu Arg Glu Ser Pro Ser Ser Asp Ser Val Thr Thr Asn  
 225 230 235 240  
 20 Thr Ser Ser Glu Thr Ala Glu Arg Gly Asp Gln Ser Ser Leu Ala Asn  
 245 250 255  
 Ser Pro Pro Glu Ala Lys Arg Gln Arg Phe Ser Val  
 260 265

## CLAIMS

We claim:

1. A transgenic flowering plant comprising in its genome a genetic construct comprising an AGL15 sequence and a promoter  
5 that promotes expression of the AGL15 sequence in the plant, the promoter not being natively associated with the AGL15 sequence.
2. The plant of Claim 1, wherein the construct comprises the AGL15 sequence of SEQ ID NO:1.
- 10 3. The plant of Claim 1, wherein the construct comprises in 5' to 3' order a CaMV 35S promoter, the AGL15 sequence of SEQ ID NO:1, a nopaline synthase terminator, and a kanamycin resistance marker.
4. A transgenic seed of a flowering plant, wherein the  
15 seed comprises in its genome a genetic construct comprising an AGL15 sequence and a promoter that promotes expression of the AGL15 sequence in flowering plants, the promoter not being natively associated with the AGL15 sequence.
5. The seed of Claim 4, wherein the construct comprises  
20 the AGL15 sequence of SEQ ID NO:1.
6. The seed of Claim 4, wherein the construct comprises in 5' to 3' order a CaMV 35S promoter, the AGL15 sequence of SEQ ID NO:1, a nopaline synthase terminator, and a kanamycin resistance marker.



7. A transgenic plant cell of a flowering plant, wherein the plant cell comprises in its genome a genetic construct comprising an AGL15 sequence and a promoter that promotes expression of the AGL15 sequence in flowering plants, the promoter not being natively associated with the AGL15 sequence.

8. The plant cell of Claim 7, wherein the construct comprises the AGL15 sequence of SEQ ID NO:1.

9. The plant cell of Claim 7, wherein the construct comprises in 5' to 3' order a CaMV 35S promoter, the AGL15 sequence of SEQ ID NO:1, a nopaline synthase terminator, and a kanamycin resistance marker.

10. A genetic construct comprising an AGL15 sequence and a promoter that promotes expression of the sequence in flowering plants, the promoter that not being natively associated with the AGL15 sequence.

11. The genetic construct of Claim 10, wherein the AGL 15 sequence is SEQ ID NO:1.

12. The genetic construct of Claim 10, wherein the promoter comprises the CaMV 35S promoter and the AGL15 sequence comprises SEQ ID NO:1.

13. The genetic construct of Claim 12 additionally comprising a nopaline synthase terminator and a kanamycin resistance marker.

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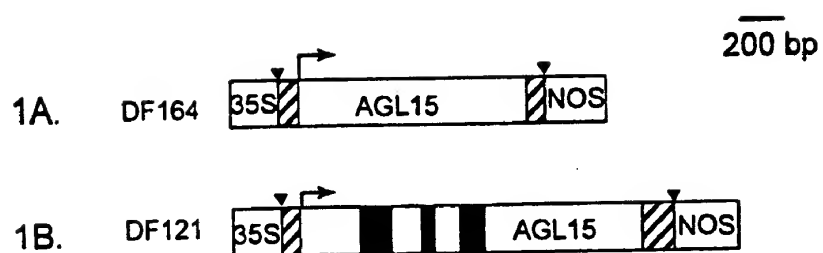


Fig. 1

# INTERNATIONAL SEARCH REPORT

Inventor: 1 Application No  
PCT/US 97/19109

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/29 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HECK G. ET AL.: "AGL15, a MADS domain protein expressed in developing embryos" THE PLANT CELL, vol. 7, no. 8, August 1995, pages 1271-1282, XP002053995 cited in the application * see the whole document, esp. p.1278, Discussion-last paragraph *	1-13
A	ROUNSELEY S. ET AL.: "Diverse roles for MADS box genes in Arabidopsis development" THE PLANT CELL, vol. 7, no. 8, August 1995, pages 1259-1269, XP002053996 cited in the application see the whole document --- -/-	1-13

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"Δ" document member of the same patent family

Date of the actual completion of the international search

30 January 1998

Date of mailing of the international search report

13/02/1998

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Kania, T

# INTERNATIONAL SEARCH REPORT

Inte      al Application No  
PCT/US 97/19109

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 11566 A (UNIV WASHINGTON) 25 April 1996 see the whole document ---	1-13
A	FLANAGAN C. AND MA H.: "Spatially and temporally regulated expression of the MADS-box gene AGL2 in wild-type and mutant arabidopsis flowers" PLANT MOLECULAR BIOLOGY, vol. 26, no. 2, October 1994, pages 581-595, XP002053997 cited in the application see the whole document ---	1-13
A	EP 0 409 628 A (CALGENE INC) 23 January 1991 cited in the application see the whole document ---	1-13
A	WO 96 21027 A (ASGROW SEED CO ;BOESHORE MAURY L (US); DENG ROSALINE Z (US); CARNE) 11 July 1996 see the whole document ---	1-13
A	WO 95 01439 A (CALIFORNIA INST OF TECHN) 12 January 1995 see the whole document -----	1-13